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*Marketta Tehikoski*  
Marketta Tehikoski  
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Osoite: Arkadiankatu 6 A Puhelin: 09 6939 500  
P.O.Box 1160 Telephone: + 358 9 6939 500  
FIN-00101 Helsinki, FINLAND

Telefax: 09 6939 5328  
Telefax: + 358 9 6939 5328

## Non-competitive immunoassay for small analytes

### Field of the Invention

The present invention relates to immunoassays and especially to  
5 non-competitive immunoassays for small analytes. The present invention further relates to reagent pairs and test-kits useful in the assays, as well as to the use of the reagent pairs and to a process for their preparation. Novel reagents and means for their preparation are also provided.

### 10 Technical Background of the Invention

In a competitive immunoassay, external reagent that competes with the analyte has to be added, which is not the case in the non-competitive assay format. The method of choice for the detection of analytes by immuno-chemistry is nowadays a non-competitive immunoassay, where two antibodies  
15 bind to two different epitopes of the analyte creating a so-called sandwich-type assay. Such an assay is well suited for high molecular weight analytes and it provides improved speed, sensitivity, and specificity, which are needed in modern immunoassays. However, it has been a difficult task to develop non-competitive assays for small analytes, because low molecular weight molecules are not large enough for binding simultaneously to more than one antibody independently. Therefore, despite many fundamental problems with respect to specificity and sensitivity, the competitive immunoassay format has  
20 been almost exclusively used for the detection of small analytes.

However, there are few publications where the development of a  
25 non-competitive immunoassay for a small analyte has been reported. In these papers, a secondary anti-immune complex (anti-IC) antibody, which binds primary anti-analyte antibody that is combined with the analyte but which does not bind the primary antibody or the analyte alone, has been developed (Ullman et al., 1993; Self et al., 1994; Towbin et al., 1995). Ullman et al.,  
30 1993, describe an antibody that recognizes an immune complex of an antibody to tetrahydrocannabinol (THC). The anti-IC antibody was obtained by using an affinity labelled anti-THC antibody as immunogen and selecting an anti-IC antibody the binding of which was enhanced by the presence of  $\Delta^9$ THC. Self et al., 1994, used the same principle in preparing anti-IC antibodies for determining digoxin. Towbin et al., 1995, report a sandwich immunoassay for the hap-  
35 ten angiotensin II, wherein the immunisation involves tolerization with uncom-

plexed primary antibody prior to immunisation with the anti-immune complex to obtain the anti-IC antibodies. The anti-IC antibodies used in non-competitive immunoassays for small analytes have so far been conventional polyclonal or monoclonal antibodies obtained by immunisation, and the assays described  
5 here include labelling of the primary antibody and immobilisation of the secondary or vice versa.

So-called 'idiometric' non-competitive immunoassay for small analytes has been developed by Mares *et al.*, 1995. They used two types of anti-idiotypic antibodies, which recognize different epitopes within the hypervariable  
10 region of the oestradiol specific primary antibody. The first anti-idiotypic antibody (betatype) possesses the capacity of competing with the analyte for an epitope at the binding site of the primary antibody. The second anti-idiotypic (alphatype) recognizes an epitope within the variable region of the primary antibody and the binding is not sensitive to the presence of the analyte. The al-  
15 phatype is, however, sterically hindered from binding to the primary antibody in the presence of the betatype. These three types of antibodies permit the development of a non-competitive assay for small analytes.

So-called open sandwich immunoassays have been developed for the detection of haptens (Suzuki *et al.*, 2000; Suzuki *et al.*, 1999; Yokozeki *et al.*, 2002). They are non-competitive assays based on a phenomenon accord-  
20 ing to which the association of separated  $V_H$  and  $V_L$  chains in some antibodies is strongly favoured in the presence of antigen (Ueda *et al.*, 1996).

Despite the significant benefits of the non-competitive immunoassay format, only few examples of that kind of assays for small analytes have been  
25 reported. The reason for that is most probably the difficulty of producing secondary (anti-immune complex, or anti-idiotypic) antibodies by immunising animals. For example anti-hapten monoclonal antibodies, which have been developed by hybridoma technology (Kohler and Milstein, 1975), are self-antigens for mice and raising immunoreponse against them is difficult  
30 (Maruyama *et al.*, 2002; Ullman *et al.*, 1993; Kobayashi *et al.*, 2000). A further problem is that the immune complex used for immunisation tends to break down before the response to the immune complex is obtained (Ullman *et al.*, 1993; Kobayashi *et al.*, 2000).

The present invention now provides a non-competitive immunoas-  
35 say protocol for small analytes, which circumvents the immunisation of animals with the immune complex, which has been so far the most challenging task

when anti-IC antibodies have been developed. The invention also facilitates a homogenous immunoassay, which further improves the speed, sensitivity and simplicity of the assay.

## 5 **Summary of the Invention**

The difficulties associated with raising anti-IC antibodies for use in immunoassays for small analytes can now be avoided by providing the necessary anti-IC antibodies from a display recombinant binding partner library instead of from immunised animals. A phage display antibody library may be  
10 constructed, which contains a vast number of clones, from which those coding the desired binding partners, such as antibody fragments, can be enriched and selected through sequential panning. This protocol opens new possibilities for developing rapid, reliable and simple immunoassays for small analytes in a cost-effective and feasible way.

15 Consequently, one object of the present invention is a non-competitive immunoassay for a small analyte comprising reacting a sample containing said analyte with a reagent pair comprising a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner. The immunoassay is characterized in that the second binding partner is obtained from a display recombi-  
20 nant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner, and determining the binding of the second binding partner thus indicating the presence of the analyte in the sample.

25 Another object of the invention is a reagent pair for a non-competitive immunoassay for a small analyte, comprising a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, characterized in that the second binding partner is obtained from a display recombinant binding partner  
30 library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

Still another object of the present invention is a test kit for a non-competitive immunoassay for a small analyte, said kit comprising a reagent pair comprising a first binding partner that binds to said analyte, and a second  
35 binding partner that binds to the complex of said analyte and said first binding partner, characterized in that the second binding partner is obtained from a

display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

5 The invention is also directed to the use of a reagent pair comprising a first binding partner that binds to an analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, in a non-competitive immunoassay for a small analyte, whereby the second binding partner is obtained from a display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

10 A still further object of the invention is a process for preparing a reagent pair for a non-competitive immunoassay for a small analyte comprising providing a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, characterized in that the second binding partner is obtained from a display  
15 recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

The invention also provides novel recombinant binding proteins, characterized in that they comprise the ligand-binding portion of M1 Fab comprising SEQ ID NO 1 and SEQ ID NO 2; M2 Fab comprising SEQ ID NO 3 and  
20 SEQ ID NO 4; or K11 scFv comprising SEQ ID NO 5.

DNA encoding the novel binding proteins as well as host cells expressing them are also included in the invention.

Advantageous embodiments of the invention are set forth in the dependent claims.

25 Other objects, details and advantages of the present invention will become apparent from the following drawings, detailed description and examples.

### **Brief Description of the Drawings**

30 Figure 1. A competitive ELISA using M1 Fab. Urine+ is urine spiked with 1 µg/ml of morphine.

Figure 2. A non-competitive time resolved fluorescent immunoassay (TR-FIA) with the Eu labelled anti-M1+morphine immune complex scFv fragment K11. a) Cross-reactivity and sensitivity of the assay. b) Sensitivity of the  
35 assay with S1 urine control dilutions as samples.

Figure 3. A homogenous TR-FRET immunoassay of morphine.

### Detailed Description of the Invention

The reagent pair for the non-competitive immunoassay of the invention comprises a first binding partner and a second binding partner. The first binding partner binds to the analyte to form a complex between the first binding partner and the analyte. The second binding partner binds to the complex formed by the first binding partner and the analyte. The binding partners are usually proteins such as antibodies including antibody fragments that have the desired binding properties. An antibody is an immunoglobulin molecule and it can belong to any of classes IgG, IgM, IgE, IgA or IgD; IgG and IgM being the most frequently used. Preferably the binding partners are antibody fragments comprising the ligand-binding site, such as Fab, or scFv fragments. The fragment known as the Fab fragment (fragment antigen binding) consists of the variable and constant domain of an immunoglobulin light chain covalently attached by a disulfide bridge to the variable and first constant domain of an immunoglobulin heavy chain. Fv (variable domain) means the variable regions of the immunoglobulin molecule that are responsible for the ligand binding. ScFv (single chain Fv) means a molecule wherein the variable domains of the heavy and light chain of an antibody are linked by a peptide to form a single polypeptide chain synthesized from a single mRNA molecule. Ligand is the substance to which the binding partner binds, in connection with antibodies it is an antigen or a hapten.

The first binding partner may be a conventional polyclonal or monoclonal antibody or fragment thereof, but preferably it is a recombinant one, as is the second binding partner. When the first binding partner has been selected for, it is complexed with its ligand and this complex is used to select for the second binding partner from a recombinant library. The first binding partner without the ligand is used as contraselection. The second binding partner should only recognise complexes, not free first binding partner nor free antigen to any significant extent.

The recombinant binding partner library is conveniently an expression library, which is typically a display library. The general principle of the display recombinant binding partner libraries is that they present the binding partner as a fusion protein on the surface, which may be the surface of a microbial cell such as a yeast or bacterial cell, or a phage. The display recombinant binding partner library can also be a display library, where stable complexes of nascent protein and mRNA are produced in an *in vitro* expression system.

Phage display libraries are the most frequently used. Antibody phage display technology and its applications are described e.g. in Hoogenboom *et al.*, 1998.

5 A phage display antibody library may be constructed by cloning immunoglobulin domains coding cDNAs into an appropriate phage display vector. DNA encoding for millions of variants of antibody fragments is batch-  
10 cloned into the vector as part of the phage coat protein. Large libraries containing millions of antibody fragments with different specificities can be obtained by transforming the vectors in bacteria. Cultivation of the bacteria leads to the expression of phages displaying antibody fragments on their surface. The gene  
15 for the displayed antibody is carried in the phage genome, thus linking genotype with phenotype. The physical linkage between the displayed protein and its DNA allows screening of vast numbers of variants of the protein, each linked to its corresponding DNA, by a simple *in vitro* selection procedure called panning. In its simplest form, panning is carried out by incubating the pool of  
20 phage-displayed variants with the ligand of interest that has been immobilized on a carrier, washing away unbound phage, and eluting specifically bound phage by disrupting the binding to the ligand. The eluted phage is then amplified *in vivo*. The process is repeated several times, resulting in stepwise enrichment of the phage pool in favour of the tightest binding sequences. After  
25 about 3 to 6 rounds of selection and amplification, the best clones are sequenced and transformed into a host cell for further expression. The host cell may be a eucaryotic or procaryotic cell e.g. a yeast, animal, plant or insect cell or bacterial cell. It may even be a hybridoma cell, which after transformation produces a recombinant monoclonal antibody. The recombinant binding partner or at least part of it may also be produced synthetically.

The concept to use recombinant antibody libraries makes the non-competitive sandwich assay for small analytes feasible. The sandwich can be detected by all the standard immunoassays. Usually one partner is immobilized on a carrier, such as a microtiter well or a bead. A sandwich is formed in  
30 the presence of analyte and the other binding partner. The sandwich may be detected e.g. by using secondary antibodies or by labelling at least one of the binding partners. The label can be any conventional label, such as a radioactive label, an enzyme, or a fluorescent compound. The assay can be e.g. ELISA or FIA.

35 A great advantage of the reagent pair of the present invention is that it enables a homogenous non-competitive immunoassay, i.e. an immunoassay

that is carried out in solution. The avoidance of immobilising and washing steps makes the assay extremely simple. Such a test is also suitable for testing on-site i.e. in places elsewhere than the laboratory.

A preferred homogenous immunoassay is one based on fluorescence resonance energy transfer (FRET), for review see Szöllösi *et al.*, 1998. In FRET, energy from a molecular fluorophore (donor) is excited to a high-energy state and transferred to another fluorophore (acceptor) via intermolecular dipole-dipole coupling. This is possible only if the distance between the donor and the acceptor is short (10-100 Å) and the fluorescence spectrum of the donor and the absorption spectrum of the acceptor partially overlap. The energy transfer is then detected as a change in fluorescence. Often time-resolved fluorescence is utilized (Hemmilä *et al.*, 1988).

FRET is applied to the present invention by labelling the two binding partners, which preferably are antibody fragments, with fluorophores that form a FRET donor-acceptor pair. When the binding partners and the analyte are small the fluorophores come into very close proximity, and a measurable FRET signal is obtained.

The invention provides a convenient and rapid analytical tool for low molecular weight analytes, such as therapeutic and abused drugs, steroids, hormones, metabolites, and environmental pollutants and toxins. A common feature of these small analytes is that they are too small for conventional sandwich assays where two antibodies recognizing different epitopes of the antigen are used. The molecular weight of these small analytes are normally less than 5000, but the limits are not absolute.

The immunoassay may be employed in all kinds of investigations, such as in detecting environmental hazards, toxic compounds in food and feed, chemicals indicative of ongoing processes e.g. of microbial processes in buildings, metabolic processes of living organisms, and in clinical tests, drug monitoring and pharmacological research. The assays are extremely suitable for detecting drugs of abuse, such as opiates (e.g. morphine), amphetamines, cannabinoids (e.g. tetrahydrocannabinol (THC)), barbiturates, benzodiazepines, cocaine, LSD, methadone, methaqualone, phencyclidine, propoxyphene, tricyclic antidepressants. The homogenous assay provides an excellent and convenient tool for on-site tests e.g. to be used by the police in raiding drivers etc. The sample to be analysed for e.g. drugs and abused drugs may be any body fluid sample, such as blood, serum, urine or saliva.



The reagent pair of the invention may be included in a test-kit. This test-kit may further comprise any other reagents needed for the assay, such as reaction solutions, buffers, washing solutions and detecting means, such as labels and optionally a fluorometer. Preferably the test-kit comprises multiple  
5 reagent pairs physically separated from each other, e.g. many in the form of a microarray, whereby e.g. many different drugs of abuse may be tested simultaneously from a single saliva sample.

In a special embodiment of the invention, a reagent pair for detecting morphine is produced and employed in a homogenous immunoassay for  
10 said substance. The first binding partner is a Fab fragment obtained from a phage display antibody library produced from cDNA from a mouse immunized with morphine conjugated to an immunocarrier BSA. Morphine specific antibody phages were enriched by selecting those binding to morphine conjugated BSA and sorting out those binding to BSA alone. After several panning rounds  
15 two high binding clones are sequenced and expressed. The expressed Fab fragments were named M1 Fab and M2 Fab.

The second binding partner is obtained from a naive scFv antibody fragment phage display library by selecting antibodies that bind to a complex of morphine and M1 Fab. First the phages are preincubated to bound M1 Fab  
20 to sort out those binding to M1 Fab as such. The unbound phages are separated and incubated with a mixture of morphine and immobilised M1 Fab to select the phages that bind to the immunocomplex. Unbound phages are washed away, and then those bound to the complex are eluted. The background is monitored by checking the binding to M1 Fab in the absence of morphine. After  
25 several panning rounds a number of clones are picked up, sequenced and expressed resulting in scFv fragment K11.

A fluorescence-based immunoassay is performed using M1 Fab labelled with europium as a first binding partner, and scFv fragment K11 labelled with Cy5 as a second binding partner. The binding partners are incubated with  
30 saliva or urine samples containing morphine and then fluorescence is measured after a predetermined time. The assay is completely homogeneous and the signal is readable in about 5 min. The sensitivity for both urine and saliva is clearly higher than that demanded by the authorities in the case of morphine, our model analyte. The performance of the test is such that the reagents are in  
35 the well of a microtiter plate and dilution series of either saliva or urine is added. In a preferred mode for e.g. police field use, the reagents are in dry

form in a vessel. Saliva is added, which dissolves the reagents and the result can be read without further processes.

The novel recombinant binding proteins provided by the invention comprise any ligand-binding portion of M1 Fab, M2 Fab or K11 scFv. By  
5 "ligand-binding portion" is meant that part of the molecule that is responsible for the binding. Minor variations or modifications of the sequences set forth in the description and claims are still within the scope of the invention provided that they do not affect the binding activity of the proteins.

The invention is illustrated by the following non-limiting example. It  
10 should be understood, however, that the embodiments given in the description above and in the example are for illustrative purposes only, and that various changes and modifications are possible within the scope of the invention.

## Example

15

### Development of an anti-morphine antibody

#### Immunisation of mice

Four six-week-old female Balb/c mice were immunised in three-week intervals with morphine conjugated BSA (Fitzgerald) in Freund's adjuvant. Serum samples were tested after second booster and the mouse showing the best response against the antigen in direct ELISA was selected to be  
20 the source of an antibody phage display library.

#### Construction of the antibody phage display library

All basic recombinant DNA methods were performed essentially as  
25 described (Sambrook et al. 1990). The mouse with the highest antibody response to morphine-BSA conjugate was sacrificed and the total RNA was isolated from the spleen cells using the RNagents® Total RNA Isolation System (Promega Co., WI, USA). The mRNA pool of the total RNA was isolated with the Oligotex mRNA Kit (QIAGEN Inc., Germany). The cDNA was synthesised  
30 from the mRNA with oligo-dT priming. Genes encoding antibody Fab fragments were amplified with PCR using antibody kappa light chain and heavy chain variable region and constant region specific primers. Antibody light chain PCR products were pooled and digested with NheI and Ascl restriction enzymes, purified by preparative agarose gel in combination with the QIAquick  
35 Gel Extraction Kit (QIAGEN Inc., Germany). The agarose gel purified antibody light chain DNA was ligated into the Fab phagemid vector phagemid9 derived

from pComb3 (Barbas *et al.*, 1991) and transformed into the *E.coli* XL1-Blue strain (Stratagene) by electroporation. Plasmid DNA was isolated with the QIAGEN Plasmid Midi Kit (QIAGEN Inc., Germany) from the overnight culture. PCR products encoding the Fd region (variable and first constant region) of the heavy chain were pooled and digested with SfiI and NotI restriction enzymes, purified by preparative agarose gel isolation and ligated to the phagemid vector containing the light chain DNA. The phagemid vector encoding both the heavy and light chain of the Fab fragment was transformed into the *E.coli* TOP10F' bacteria (Invitrogen Inc., CA, USA) by electroporation. Transformed bacteria were incubated over night at +37°C on a shaker and plasmid DNA was isolated with the QIAGEN Plasmid Midi Kit. The diversity of the antibody library was ensured by sequencing partial V<sub>L</sub>-or V<sub>H</sub>-gene regions of individual clones.

A phage display antibody library was made as follows: 4 µg of antibody library plasmid DNA was transformed into *E.coli* TOP10F' by electroporation in two parallel transformations. After transformations, cells were suspended into 2,8 ml of SOC medium and incubated for 1 h at +35°C on a shaker. 7 ml of prewarmed (+37°C) SB medium, 20 µg/ml of carbenicillin, and 10 µg/ml of tetracycline was added. After 1-h incubation at +35°C on a shaker, 30 µg/ml of carbenicillin was added and the incubation was continued for 1 h after which 1 ml (~10<sup>11</sup> pfu) of helper phage VCS-M13 (Stratagene) was added and the phages were let to infect the bacteria for 20 min at +35°C with a slow shaking. The parallel transformations were joined together and 80 ml of prewarmed (+37°C) SB medium with 50 µg/ml carbenicillin and 10 µg/ml of tetracycline was added. After 2-h incubation at +35°C on a shaker, 70 µg/ml of kanamycin was added and the incubation was continued over night.

Cells were centrifuged for 15 min at 4000g at +4°C. 20 ml of 20% PEG, 2.5 M NaCl (PEG/NaCl) was added to the supernatant and it was incubated for 30 min on ice. PEG precipitated phages were centrifuged for 20 min at 13000g at +4°C. The pellet was suspended in 2 ml of PBS and 1 ml was transferred into two eppendorf tubes. After centrifugation for 5 min at +4°C, phages were precipitated by adding 200 µl of PEG/NaCl to the supernatant. The solution was mixed and centrifuged for 5 min at +4°C. The pellet was suspended in 1 ml of PBS or PBS+1% BSA. 1 µl of 20% Na-azide was added as a preservative. The phage display antibody library was stored at +4°C.

### Selection of the anti-morphine library

Morphine specific antibodies were selected from the phage display antibody library by the following selection procedure: A microtiter well was coated over night at +4°C with 1 µg of morphine conjugated BSA (morphine-  
5 BSA; Fitzgerald Industries International, Inc., MA, USA) in 100 µl of 100 mM Na-bicarbonate buffer, pH 9.8. The well was washed two times with PBS and blocked with 1% BSA in PBS for 1 h at +37°C. 100 µl of the phage library was incubated in the well for 1 h at RT with shaking, after which the unbound  
10 phages were removed and the well was washed 22 times with PBS. Bound phages were eluted with 100 µl of 100 mM HCl (pH 2.2) for 15 min. Eluted phages were removed from the well and neutralised with 1 M Tris. 3 ml of fresh *E.coli* XL1-Blue cells (OD<sub>600</sub>≈1) grown in SB supplemented with 10 µg/ml of tetracycline was infected with eluted phages at +35°C for 15 min. 7 ml of pre-warmed SB (+37°C) with 20 µg/ml of carbenicillin and 10 µg/ml of tetracycline  
15 was added and the culture was incubated for 1 h at +35°C on a shaker. 30 µg/ml of carbenicillin was added and incubation was continued for 1 h. 1 ml of helper phage VCS-M13 was added to the culture and incubated at +35°C for 15 min with a slow shaking. The culture was diluted with 90 ml of prewarmed (+37°C) SB with 50 µg/ml carbenicillin and 10 µg/ml tetracycline. After 2 h in-  
20 cubation at +35°C on a shaker, 70 µg/ml of kanamycin was added and the incubation was continued over night. The purification of the amplified phages was performed by PEG precipitation as described above.

The purified phages were used in further enrichment rounds. After four selection rounds, morphine specific antibody phages had been enriched  
25 over 1000 times when compared to the background. The background binding of the phages was monitored in parallel in each selection round by incubating the phage library in a BSA coated microtiter well. The well was washed and the phages were eluted as in the morphine-BSA coated well. The enrichment of the morphine specific phages was monitored by comparing the amount of the  
30 eluted phages from the morphine-BSA coated well to the amount of eluted phages from the background well.

### Characterisation of individual clones

After the fourth panning round, the phagemid DNA was isolated with the QIAGEN Plasmid Midi Kit. The plasmid was digested with NheI and NotI  
35 restriction enzymes to isolate the Fab gene fragment. The agarose gel isolated

DNA was ligated to the expression vector pKKtac. The ligation reaction was transformed into the *E.coli* XL1-Blue cells.

Individual clones were picked and miniprep DNA was extracted with the QIAprep Spin Miniprep Kit (QIAGEN Inc., Germany). According to sequencing of the minipreps, two different Fab clones were found. These clones were named M1 and M2. The amino acid sequences of the M1 Fab fragment were SEQ ID NO 1 (light chain) and SEQ ID NO 2 (heavy chain). The amino acid sequences of M2 Fab fragment were SEQ ID NO 3 (light chain) and SEQ ID NO 4 (heavy chain). Small-scale (3 ml) Fab expression cultures were also made. Periplasmic fraction of the cells was isolated by freezing and thawing the cells for three times in PBS.

The binding of individual Fab clones to morphine was tested by ELISA in morphine-BSA coated microtiter wells: Microtiter wells were coated with 200 ng of morphine-BSA in 0.1 M Na-bicarbonate buffer, pH 9.8 for over night at +4°C. Control wells were filled only with the buffer. After coating, wells were washed three times with PBS and blocked with 0.5% BSA in PBS (BSA/PBS) for 1 h at RT. Wells were washed three times with PBS and 1:10 dilution of the periplasmic fractions was added into wells in 100 µl of BSA/PBS. After 1-h incubation at RT on a shaker, wells were washed three times with PBS and 1:2000 diluted alkaline phosphatase conjugated anti-mouse Fab specific antibody (Sigma, A-1293) was added in 100 µl of BSA/PBS. The wells were incubated for 1 h at RT on a shaker and washed three times with PBS. 100 µl of alkaline phosphatase substrate solution (2 mg/ml of p-nitrophenylphosphate di-Na-salt in diethanolamine-MgCl-buffer) was added to the wells and absorbance was measured. According to the preliminary results, the M1 Fab showed better performance in the assay and was therefore chosen for the continuation.

#### Fermentation and purification

The anti-morphine Fab fragment M1 in the expression vector pKKtac was transformed in the *E.coli* expression strain RV308. The Fab was expressed by fed-batch fermentation in a Bio-Flow IV fermenter (New Brunswick) and purified by the Sepharose SP ion-exchange and protein G chromatography (Pharmacia).

#### Performance of the primary antibody in a competitive immunoassay

The performance of the M1 anti-morphine Fab fragment was tested in a competitive ELISA assay using the commercial urine toxicology controls

S1 and S3 (Bio-Rad). The S1 urine toxicology control contains drugs and drug metabolites (including morphine) at concentrations 20-25% below immunoassay cut-off levels as recommended by the U.S. Substance Abuse and Mental Health Services Administration (SAMSHA) and other agencies. The S3 urine  
5 toxicology control contains drugs and drug metabolites at concentrations approximately three times immunoassay cut-off levels. Microtiter wells were coated over night at +4°C with 500 ng of morphine-BSA in 100 µl of Na-bicarbonate buffer, pH 9.8. Wells were washed three times with PBS and blocked with BSA/PBS for 1 h at RT. After three washes with PBS, S1, S3  
10 (Bio-Rad), positive control, or negative control urine dilutions were added to the wells in 100 µl of BSA/PBS spiked with 1 ng of M1 Fab. Wells were incubated for 2 h at RT on a shaker and washed three times with PBS. 1:2000 diluted alkaline phosphatase conjugated anti-Fab antibody (Sigma, A-1293) was added to the wells in 100 µl of BSA/PBS and incubated for 1 h at RT on a  
15 shaker. Wells were washed three times with PBS, 100 µl of alkaline phosphatase substrate solution was added and  $A_{405}$  was measured. Results are shown in Figure 1. A positive result could be achieved with 1:64 dilution of the sample.

## 20 **Development of an anti-immune complex antibody specific to the immune complex of M1 Fab and morphine**

### Selection of the immune complex specific antibody from a naive human scFv phage display library

M1 Fab fragment was biotinylated with ImmunoPure Sulfo-NHS-LC-Biotin Kit (Pierce). Biotinylated antibody was purified and buffer was changed  
25 to PBS with Econo-Pac 10DG Columns (Bio-Rad, CA, USA). 200 µl of a naive human scFv phage display library (Kappa or Lambda light chain) in BSA/PBS was preincubated with 10 µl of streptavidin coated magnetic beads (Dyna, M-280) and 0.5 µg of biotinylated M1 Fab for over night at +4°C. The naive human scFv phage display library was constructed from pooled lymphocytes of  
30 50 healthy individuals. The size of the library was estimated to be  $1 \times 10^8$  clones. The naive human scFv phage display library contains the IgM specific  $V_H$ -genes combined either with the kappa or lambda specific  $V_L$ -genes. Unbound phages were separated from the beads and 100 µl of them was incubated with 100 ng of morphine, 500 ng of biotinylated M1 Fab, and 5 µl of  
35 streptavidin coated magnetic beads for 1 h at RT on a shaker. The background

for the selection procedure was implemented in a similar way but omitting the morphine from the binding reaction. Magnetic beads were washed five times with 0.5 ml of PBS and bound phages were eluted with 100 µl of HCl (pH 2.2) for 30 min. The eluted phages were neutralised with 1M Tris and *E.coli* XL1-Blue cells were infected. Cells were grown and phages were purified as described previously. After five panning rounds enrichment was seen with the scFv library having the kappa light chains, when the amount of eluted phages was compared to the amount of eluted phages from the background control well. The enrichment of specific binders to the immune complex, formed by M1 Fab and morphine, was also clearly seen in a phage ELISA when the eluted phage pools from the selection rounds were tested.

#### Characterisation of individual clones

Individual phage clones were picked and they were sequenced. All of the clones had the same sequence (SEQ ID NO 5). The amino acid sequence of anti-M1+morphine immune complex scFv fragment was named K11 scFv.

#### Expression and purification

The gene encoding the scFv fragment K 11 was inserted into the expression vector pKKtac and transformed into the *E.coli* RV308 strain. Cells were inoculated into 20 ml of LB with 100 µg/ml ampicillin and were incubated over night at +37°C on a shaker. From the overnight culture a 10 ml inoculate was added into two erlenmeyer bottles containing 500 ml of LB with 100 µg/ml of ampicillin. Cells were incubated at +37°C on a shaker until the OD<sub>600</sub> was 1 after which 0.5 ml of 1M IPTG and 100 µg/ml of ampicillin were added into the culture and the incubation was continued over night at +30°C on a shaker. Both the supernatant of the culture medium and the periplasmic fraction of the cells were used for the purification of the K11 scFv. Cells were centrifuged at 4000g for 15 min and the supernatant was poured into a clean flask. The cells were resuspended in 20 ml of PBS. The periplasmic fraction of the cells was isolated by freezing (-70°C) and thawing (+37°C) the cells for three times. After centrifugation at 12000g for 30 min, the clear periplasmic supernatant was taken. The culture medium and the periplasmic fraction were treated with 2 mg/ml of DNaseI to remove residual chromosomal DNA for two hours at +37°C. Since the expressed K11 scFv has a 6xHis-tag in its C-terminus, it could be purified by immobilised metal affinity chromatography (IMAC). The K11 scFv was purified by expanded bed chromatography using STREAMLINE

Chelating (Amershampharmacia biotech) as a matrix and copper as the metal chelate. The purity of K11 scFv was checked with SDS-PAGE.

### **Development of a non-competitive immunoassay for morphine**

#### **5        Labelling of the anti-immune complex antibody K11 scFv with europium**

The purified anti-M1 and morphine immune complex scFv fragment K11 was labelled with europium chelate by the DELFIA Eu-Labeling Kit (Wallac) as described by the manufacturer. Buffer of the labelled K11 scFv was  
10 changed to 50 mM Tris pH 7.8, 0.9% NaCl. Labelling yield was 0.4 Eu/scFv.

#### **A non-competitive immunoassay for morphine**

The sensitivity and cross-reactivity of K11 scFv was studied by a non-competitive immunoassay. Transparent Streptawell (Roche) streptavidin coated microtiter wells were washed three times with PBS. 500 ng of biotinylated anti-morphine M1 Fab was added into the wells in 100 µl of BSA/PBS  
15 and the wells were incubated for 30 min at RT on a shaker. After three PBS washes, sample dilutions spiked with morphine, amphetamine, tetrahydrocannabinol (THC) or S1 urine control were added into the wells in 50 µl of BSA/PBS. 1:100 diluted europium labelled K11 scFv was added into the wells  
20 in 50 µl of BSA/PBS and the incubation was continued for 1 h. Wells were washed three times with PBS and 100 µl of Enhancement solution (Wallac) was added into the wells. After 15-min shaking at RT, fluorescence was detected by VictorV fluorometer (Wallac). Results are shown in Figure 2. There is a significant difference in affinity between the binding of K11 scFv to the primary antibody fragment M1 and to the M1 and morphine immune complex.  
25 This allows the detection of 1 ng/ml of morphine in a sample. No cross-reactivity with amphetamine or THC was detected.

#### **A homogenous time resolved fluorescent resonance energy transfer (TR-FRET)**

30 The anti-morphine Fab fragment M1 was labelled with europium by the LANCE Eu-W1024 ITC chelate Kit (Wallac). The buffer of the labelled M1 was changed to 50 mM Tris, pH 7.8, 0.9% NaCl. The labelling yield was 1.1 Eu/Fab. The anti-M1+morphine immune complex scFv fragment K11 scFv was labelled with Cy5 by the FluoroLink-Ab Cy5 labelling kit (Amershampharmacia  
35 biotech). The labelling yield was 2 Cy5/scFv.



Saliva was spiked with various dilutions of morphine and these samples were filtered through cotton wool before adding into the black micro-titer wells (Nunc). 1  $\mu$ g of europium labelled M1 Fab and 1  $\mu$ g of Cy5 labelled K11 scFv was added into the wells in 50  $\mu$ l of BSA/PBS. TR-FRET was read  
5 after 5, 15, 30, or 60 min incubation at RT by VictorV fluorometer (Wallac). Results are shown in Figure 3. After five minutes incubation, 1 ng/ml of morphine in saliva was detected.

While one strategy for providing reagents and assays of the invention has been described, numerous variations and modifications will become  
10 apparent to the person skilled in the art.

## References

- Barbas, C.F. III, Kang, A.S., Lerner, R.A., Benkovic, S.J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA* 88:7978-7982.
- Hemmilä, I., Malminen, O., Mikola, H., Lövgren, T. (1988) Homogeneous time-resolved fluoroimmunoassay of thyroxine in serum. *Clin. Chem.* 34:2320-2322.
- Hoogenboom H.R., de Bruijne, A.P., Hufton, S.E., Hoet, R.M., Arends, J.-W., and Roovers, R.C. (1998) Review article: Antibody phage display technology and its applications. *Immunotechnology* 4, 1-20.
- Kobayashi, N., Oiwa, H., Kubota, K., Sakoda, S., Goto, J. (2000) Monoclonal antibodies generated against an affinity-labeled immune complex of an anti-bile acid metabolite antibody: an approach to noncompetitive hapten immunoassays based on anti-idiotypic or anti-metatype antibodies. *J Immunol Methods*, 245, 95-108.
- Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256, 495-7.
- Mares, A., De Boever, J., Osher, J., Quiroga, S., Barnard, G. and Kohen, F. (1995) A direct non-competitive idiometric enzyme immunoassay for serum oestradiol. *J Immunol Methods*, 181, 83-90.
- Maruyama, H., Sperlagh, M., Zaloudik, J., Liang, S., Mizuki, K., Molthoff, C. and Herlyn, D. (2002) Immunization procedures for anti-idiotypic antibody induction in mice and rats. *J Immunol Methods*, 264, 121.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1990) *Molecular Cloning: A laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Self, C.H., Dessi, J.L. and Winger, L.A. (1994) High-performance assays of small molecules: enhanced sensitivity, rapidity, and convenience demonstrated with a noncompetitive immunometric anti-immune complex assay system for digoxin. *Clin Chem*, 40, 2035-41.
- Suzuki, C., Ueda, H., Mahoney, W. and Nagamune, T. (2000) Open sandwich enzyme-linked immunosorbent assay for the quantitation of small haptens. *Anal Biochem*, 286, 238-46.

- Suzuki, C., Ueda, H., Tsumoto, K., Mahoney, W.C., Kumagai, I. and Nagamune, T. (1999) Open sandwich ELISA with V(H)-V(L)-alkaline phosphatase fusion proteins. *J Immunol Methods*, 224, 171-84.
- Szöllösi, J., Damjanovich, S., Mátyus, L. (1998) Application of Fluorescence Resonance Energy Transfer in the Clinical Laboratory: Routine and Research. *Communications in Clinical Cytometry*, 34:159-179.
- Towbin, H., Motz, J., Oroszlan, P. and Zingel, O. (1995) Sandwich immunoassay for the hapten angiotensin II. A novel assay principle based on antibodies against immune complexes. *J Immunol Methods*, 181, 167-76.
- Ueda, H., Tsumoto, K., Kubota, K., Suzuki, E., Nagamune, T., Nishimura, H., Schueler, P.A., Winter, G., Kumagai, I. and Mahoney, W.C. (1996) Open sandwich ELISA: a novel immunoassay based on the interchain interaction of antibody variable region. *Nat Biotechnol*, 14, 1714-8.
- Ullman, E.F., Milburn, G., Jelesko, J., Radika, K., Pirio, M., Kempe, T. and Skold, C. (1993) Anti-immune complex antibodies enhance affinity and specificity of primary antibodies. *Proc Natl Acad Sci U S A*, 90, 1184-9.
- Yokozeki, T., Ueda, H., Arai, R., Mahoney, W. and Nagamune, T. (2002) A homogeneous noncompetitive immunoassay for the detection of small haptens. *Anal Chem*, 74, 2500-4.

### Claims

1. Non-competitive immunoassay for a small analyte comprising reacting a sample containing said analyte with a reagent pair comprising a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, characterized in that the second binding partner is obtained from a display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner, and determining the binding of the second binding partner, thus indicating the presence of the analyte in the sample.

2. The assay of claim 1, characterized in that the first and second binding partners are selected from antibody fragments Fab and scFv.

3. The assay of claim 1 or 2, characterized in that it is a homogeneous assay.

4. The assay of claim 3, characterized in that it is based on fluorescence resonance energy transfer (FRET).

5. The assay of any of the preceding claims, characterized in that the analyte is a drug of abuse.

6. The assay of claim 5, characterized in that the analyte is morphine, tetrahydrocannabinol (THC) or amphetamine.

7. Reagent pair for a non-competitive immunoassay for a small analyte, comprising a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, characterized in that the second binding partner is obtained from a display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

8. Test kit for a non-competitive immunoassay for a small analyte, said kit comprising a reagent pair comprising a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, characterized in that the second binding partner is obtained from a display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

9. The test kit of claim 8, characterized in that the first and second binding partners are selected from antibody fragments Fab and scFv.

10. The test kit of claim 8 or 9, c h a r a c t e r i z e d in that it comprises reagents for a homogeneous assay.

11. The test kit of claim 10, c h a r a c t e r i z e d in that it comprises reagents for a fluorescence resonance energy transfer (FRET) based assay.

12. The test kit of any of claims 8 to 11, c h a r a c t e r i z e d in that it comprises reagents for assaying a drug of abuse.

13. The test kit of claim 12, c h a r a c t e r i z e d in that it comprises multiple reagent pairs for assaying multiple drugs of abuse.

14. The test kit of any of claims 8 to 13, c h a r a c t e r i z e d in that it comprises reagents for assaying morphine, tetrahydrocannabinol (THC) or amphetamine.

15. The test kit of claim 14, c h a r a c t e r i z e d in that it comprises one or more reagents from the group consisting of the ligand-binding portion of M1 Fab comprising SEQ ID NO 1 and SEQ ID NO 2; M2 Fab comprising SEQ ID NO 3 and SEQ ID NO 4; and K11 scFv comprising SEQ ID NO 5.

16. Use of a reagent pair comprising a first binding partner that binds to an analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, in a non-competitive immunoassay for a small analyte, whereby the second binding partner is obtained from a display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

17. Process for preparing a reagent pair for a non-competitive immunoassay for a small analyte, comprising providing a first binding partner that binds to said analyte, and a second binding partner that binds to the complex of said analyte and said first binding partner, c h a r a c t e r i z e d in that the second binding partner is obtained from a display recombinant binding partner library by selecting a binding partner that binds to said complex of the analyte and first binding partner.

18. The process of claim 17, c h a r a c t e r i z e d in that recombinant antibody fragments are prepared from a phage display library.

19. The process of claim 17 or 18, c h a r a c t e r i z e d in that the first binding partner is also obtained from a display recombinant binding partner library.

20. Recombinant binding protein, c h a r a c t e r i z e d in that it comprises the ligand-binding portion of M1 Fab comprising SEQ ID NO 1 and

SEQ ID NO 2; M2 Fab comprising SEQ ID NO 3 and SEQ ID NO 4; or K11 scFv comprising SEQ ID NO 5.

21. The recombinant binding protein of claim 20, characterized in that it has the amino acid sequence SEQ ID NO 1 and SEQ ID NO 2; SEQ ID NO 3 and SEQ ID NO 4; or SEQ ID NO 5.

22. DNA characterized in that it encodes a recombinant binding protein of claim 20 or 21.

23. Host cell characterized in that it expresses a recombinant binding protein of claim 20 or 21.

**(57) Abstract**

The invention is directed to a non-competitive immunoassay for small analytes, wherein the analyte is reacted with two binding partners. The first binding partner binds to the analyte to form a complex between the first binding partner and the analyte, and the second binding partner binds to the complex formed by the first binding partner and the analyte. The resulting complex formed between the analyte and the binding partners is detected. The binding partners are proteins, such as antibodies including antibody fragments. The invention further relates to reagent pairs and test kits useful in the assays, as well as to the use of the reagent pairs and to a process for their preparation. Novel reagents and means for their preparation are also provided.

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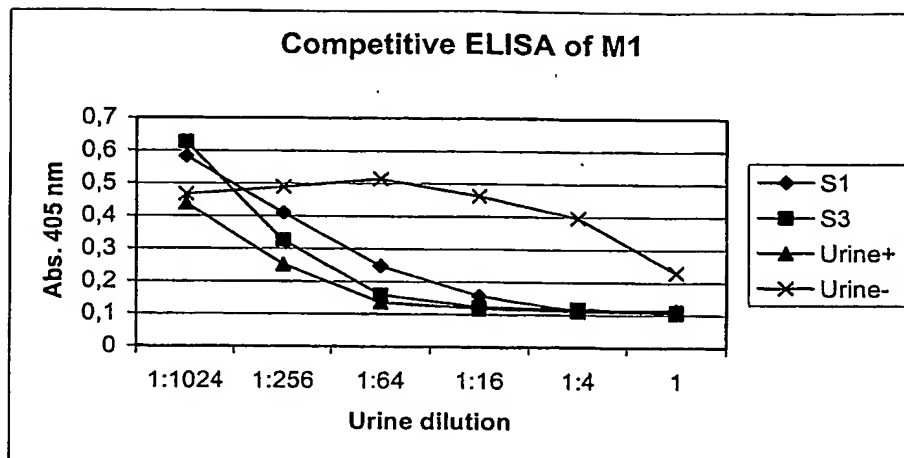
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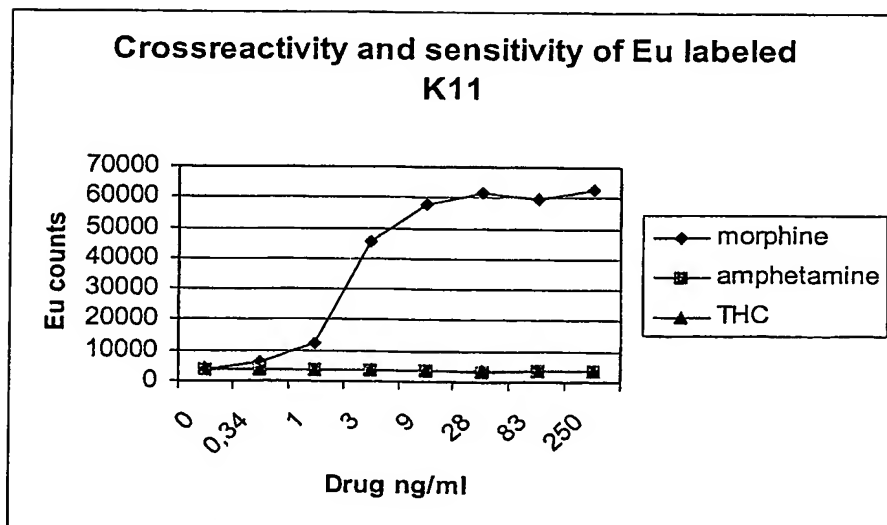
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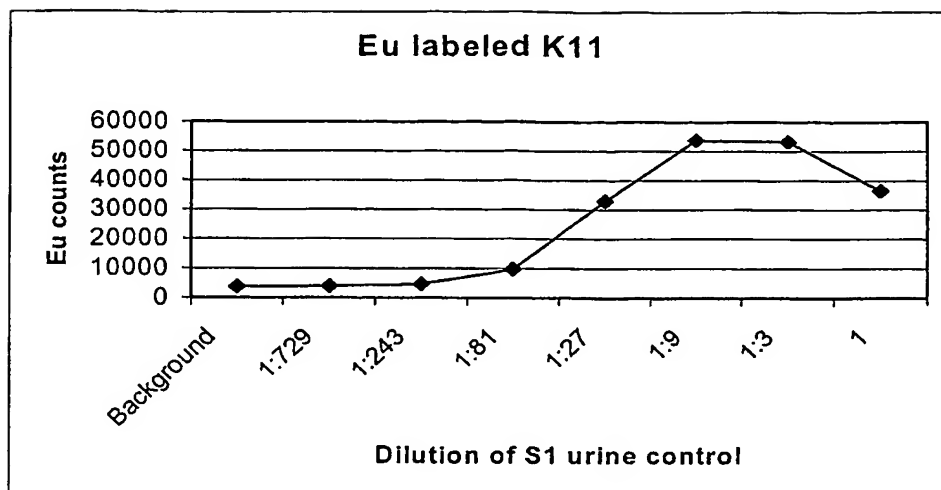
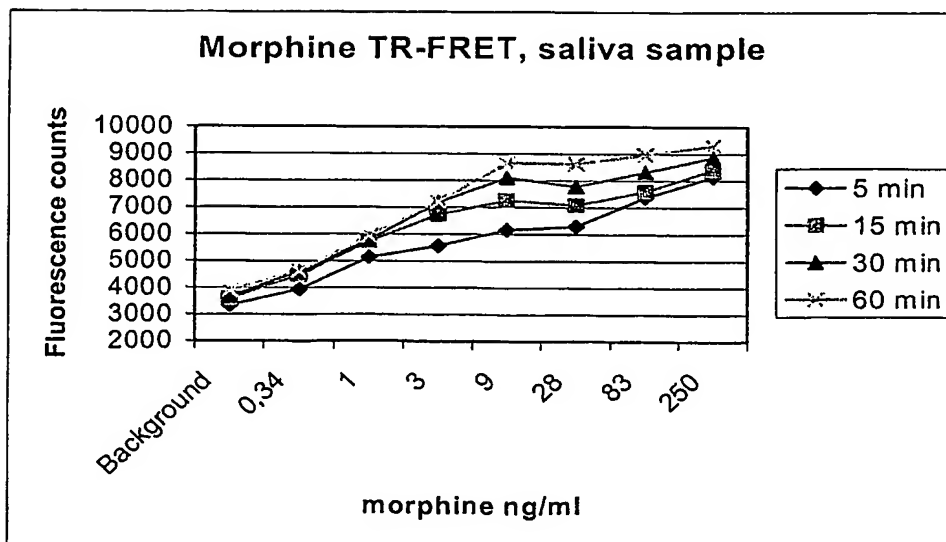
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**Figure 1**



**Figure 2a**

**Figure 2b****Figure 3**